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# A novel member of family 30 glycoside hydrolase subfamily 8 glucuronoxylan *endo*-β-1,4-xylanase (*Ct*XynGH30) from *Clostridium thermocellum* orchestrates catalysis on arabinose decorated xylans

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ABSTRACT

A thermophilic enzyme, *Ct*XynGH30 from *Clostridium thermocellum* was identified as glucuronoxylan *endo*-β-1,4-xylanase displayed activity extending to arabinose decorated xylans unlike other glucuronoxylan *endo*-β-1,4-xylanase from family 30 sub-family 8 of glycoside hydrolase. Modular *Ct*XynGH30 comprises N-terminal catalytic module *Ct*Xyn30A and C-terminal carbohydrate binding module of family 6, (*Ct*CBM6). The purified *Ct*XynGH30 displayed a molecular mass of approximately, 60 kDa. *Ct*XynGH30 showed an optimum pH 6.0 and optimum temperature 70 °C. *Ct*XynGH30 displayed maximum activity against glucuronic acid substituted beechwood xylan followed by birchwood- and 4-O-methyl glucurono-xylan. *Ct*XynGH30 also displayed activity against arabinoxylans that were also confirmed by TLC analysis. The ability of *Ct*XynGH30 to hydrolyse both glucuronic and arabinose decorated xylans distinguishes it from other enzymes of this subfamily. The $^{1}$H NMR analysis of hydrolysed products of beechwood xylan confirmed the presence of acidic *xylo*-oligosaccharides having methyl glucuronic acid moiety penultimate to the reducing end of xylan. *Ct*XynGH30 produced a range of acidic *xylo*-oligosaccharides and arabinoxylo-oligosaccharides which have potential food and health applications.



## 1. Introduction

Bioconversion of plant biomass into biofuel and several other value added products such as xylitol, organic acids and oligosaccharides is a subject of interest for many researchers [1]. The low cost and widespread availability of plant polysaccharides, which is the largest renewable organic carbon source on earth, makes it possible for these industrial applications [2]. Hemicellulose contributes to the second highest percentage after cellulose in which heteroxylans constitute a major part in land plants [3]. Heteroxylans have xylose (a pentose sugar) residues in the main chain linked with β-1,4 xylosidic bond along with substitutions of different side chains linked either directly or through their derivatives such as arabinose, glucuronic, acetic, galactose, ferulic acid and *p*-coumaric acid. The complex nature of hemicellulose requires a cumulative action of various specific xylanases to degrade it efficiently and completely [4,5]. So far characterized xylanases predominantly belong to GH10 and GH11 families, but they are also present in the families GH5, GH30 and GH43, where the families like GH5,GH10 and GH30 are the members of the same superfamily or clan i.e. GH-A (http://www.cazypedia.org/). The family GH30 members follow the retention type mechanism and contain $(\beta/\alpha)_8$ TIM-barrel fold catalytic core like the other members of GH-A clan. A distinctive structural feature associated with this family is that along with the $(\beta/\alpha)_8$ TIM-barrel catalytic core motif, it contains a side β-stand rich structure that resembles CBMs known as "side β-structure". The other distinguishing property of GH30 subfamily 8 xylanases is the requirement of 4-O-methyl-D-glucuronosyl (MeGlcA) residues as the side chains to act on a xylan main chain hence they are also called 'appendage dependant xylanases' [6].

*Clostridium thermocellum* is a thermophilic, anaerobic, rod shaped bacterium known to exhibit one of the highest rates of cellulose utilization with the help of extracellular cellulosome [7]. The full length *Ct*XynGH30 belongs to subfamily 8 of GH30 and it is a modular, cellulosomal carbohydrate active enzyme from *C. thermocellum*. *Ct*XynGH30 comprises an N-terminal catalytic module

*Abbreviations:* CBM, carbohydrate binding module; aa, amino acid; IMAC, immobilized metal-ion affinity chromatography; pNP, paranitrophenyl; TLC, thin layer chromatography; MeG, methyl glucuronic acid; $MeGX_n$/$MeGAX_n$, methyl glucuronoxylan; XOS, xylo-oligosacharide; U-XOS, uronic acid substituted *xylo*-oligosachharide.

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designated as *Ct*Xyn30A along with a C-terminal family 6 carbohydrate binding module, *Ct*CBM6 [8]. The enzyme *Ct*Xyn30A was crystallized and structurally characterized [9]. The structure and functional studies of a *Ct*CBM6 module of *Ct*XynGH30 displayed similar affinities against highly substituted xylans (wheat- and rye-arabinoxylans) as well as poorly substituted xylans (birch-, beech- and oat spelt-xylan) [10]. The production of oligosaccharides has attracted the worldwide attention because of their immense applications in the fields of medicinal, nutraceutical and in the food industries [11]. The health benefits of functional oligosaccharides have been well established and described [12]. *Xylo*-oligosaccharides (XOS) act as potent prebiotic and serve as a food additive [13]. The acidic *xylo*-oligosacharide showed antibacterial activities [14]. Glucuronic acid-containing acidic xylans have been reported as anti-cancer and suppressing the growth of tumor cells [15]. Arabinoxylo-oligosaccharides (AXOS) have been used as a prebiotics and also as a sucrose replacer in sugar-snap cookies [16,17]. In the present study, the enzyme *Ct*XynGH30 was explored and biochemically characterized. The enzyme, *Ct*XynGH30 was found unique distinguishing itself from those previously reported enzymes from family GH30, subfamily 8 in that it hydrolysed not only the glucuronic acid substituted xylans but also the arabinose substituted xylans such as arabinoxylans.

## 2. Material and methods

### 2.1. Overall modular architecture of Ct*XynGH30*

The complete nucleotide and protein sequence (GenBank Accession No: ABN54208 and UniProt ID A3DJS9) of modular *Ct*XynGH30 from *C. thermocellum* and assignment of its domain boundaries has been described earlier [8].

### 2.2. PCR amplification and cloning of gene encoding Ct*XynGH30*

Oligonucleotide primers containing *NheI* and *XhoI* restriction sites were designed.

The following set of primers encoding *Ct*XynGH30 gene (Eurofins Genomics Pvt. Ltd., India) was used for amplifying: forward primer 5′- CTCTGCTAGCGCAACAATCAACTTGTCG-3′ and the reverse primer 5′- CACACTCGAGTTACTCCTGTTTTGACTC −3′. The gene was amplified using *C. thermocellum* genomic DNA (7.7 ng) as a template. The 50 μl PCR reaction mixture contained 0.45 μM of each primer, 1x reaction buffer containing $Mg^{2+}$ ions (2.5 mM), 1.6 mM dNTP mix and 1.25 U *Pfu* DNA polymerase. The PCR cycle conditions used were as follows: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, extension at 72 °C for 120 s and the final elongation step at 72 °C for 5 min. The amplified PCR product was purified using the QIAquick gel-extraction kit and ligated to pGEM-T Easy vector (Promega, USA). The positive clones were screened by according to the manufacturer's instruction. Restriction digestion of isolated plasmid DNA of the positive clone was performed using *NheI* and *XhoI* enzymes and the gene encoding of *Ct*XynGH30 was further cloned in to pET-28a(+) expression vector (Novagen, EMD4 BioScience, Germany). The resulting recombinant plasmids were transformed using competent *Escherichia coli* (DH5α) cells. After transformation, the cells were grown on LB agar plates supplemented with kanamycin (50 mg/ml) at 37 °C. The positive clones were confirmed by restriction digestion of recombinant plasmid as well as by PCR amplification of the gene encoding *Ct*XynGH30 using gene specific primers and recombinant DNA plasmid as the template.

### 2.3. Protein expression and purification

*E. coli* BL-21(DE3) (Novagen, EMD4 BioScience, Germany) competent cells were used for the transformation of recombinant plasmid DNA. The cells harbouring the gene encoding *Ct*XynGH30 were grown in 1 l shake flask containing 200 ml LB medium supplemented with kanamycin (50 mg/ml) by incubating at 37 °C and 180 rpm until the mid-exponential phase when absorbance at 550 nm ($A_{550}$) reached ~0.6. After that the cells were induced with 1.0 mM (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG) and further incubated at 25 °C and 180 rpm for 16 h. The cells were harvested by centrifugation at 8000*g* at 4 °C for 15 min and the cell pellet was re-suspended in 10 ml lysis buffer (50 mM sodium phosphate buffer, pH 6.0, 1 mM phenyl-methylsulfonyl fluoride, 150 mM NaCl). The cells were then sonicated on ice for 15 min (8 s on/16 s off pulse, 33% amplitude, Sonics, Vibra cell) and centrifuged again at 17,000*g* at 4 °C for 40 min to obtain the cell free extract. The recombinant *Ct*XynGH30 containing N-terminal $His_6$ tag was purified from the cell-free extract by immobilized metal-ion affinity chromatography (IMAC) using a 5 ml Sepharose column (HiTrap Chelating, GE Healthcare) according to the manufacturer's instruction. The column was equilibrated with 5 vols of equilibration buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 60 mM Imidazole). The cell free extract (10 ml) was filtered through 0.45 μm membrane and loaded on to the column at a flow rate of 1 ml/min. The enzyme was eluted with elution buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 300 mM Imidazole) and 8 fractions of 1 ml each was collected. The eluted enzyme was desalted by dialysis carried at 4 °C using the 20 mM sodium phosphate buffer (pH 6.0) with 3 changes.

### 2.4. Gel electrophoresis and protein concentration estimation

The homogeneity and molecular mass of purified recombinant *Ct*XynGH30 was analyzed by 12% (w/v) SDS-PAGE [18]. The enzyme concentration was measured by using molar extinction coefficient of 91853 $M^{-1}$ $cm^{-1}$ (calculated from the sequence at ExPASy server, http://web.expasy.org/protparam/) on a NanoDrop spectrophotometer (2000c Thermo).

### 2.5. Substrates and chemicals

Natural polysaccharides like rye arabinoxylan, wheat arabinoxylan (soluble and insoluble), arabinogalactan, sugar beet arabinan, rhamnogalacturonan and carob galactomannan were purchased from Megazyme International, Ireland. Oat spelt xylan, birchwood xylan, beechwood xylan, 4-O-methyl glucuronoxylan and other hemi-cellulosic, cellulosic and pectin substrates were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., India. Synthetic substrates, *p*-nitrophenyl glycoside (*p*NP-glycosides) viz. 4-nitrophenyl β-D-xylopyranoside, 4-nitrophenyl α-D-xylopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-α-L-arabinopyranoside, *p*-nitrophenyl-β-L-arabinopyranoside, 4-nitrophenyl acetate, 4-nitrophenyl α-D-glucopyranoside, 4-nitrophenyl β-D-glucopyranoside and 4-nitrophenyl β-D-glucuronide were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., India.

*Xylo*-oligosaccharide and aldouronate standards were purchased from Megazyme International, Ireland.

### 2.6. Assay of Ct*XynGH30 with natural and synthetic* p*-nitrophenyl glycoside substrates*

*Ct*XynGH30 was assayed against several natural polysaccharides, in order to determine the specificity and the highest activity. The assays were performed in 100 μl reaction mixture using